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<b>(21) International Application Number:</b> PCT/US95/02491 <b>(22) International Filing Date:</b> 27 February 1995 (27.02.95) <b>(30) Priority Data:</b> 205,682 3 March 1994 (03.03.94) US <b>(60) Parent Application or Grant</b> (63) Related by Continuation US 205,682 (CIP) Filed on 3 March 1994 (03.03.94) <b>(71) Applicant: (for all designated States except US):</b> MERCK & CO., INC. [US/US], 126 East Lincoln Avenue, Rahway, NJ 07065 (US). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> MARTIN, Juan, F. [ES/ES]; Vegazana Campus, E-24071 Leon (ES). COQUE, Juan-Jose, R. [ES/ES]; Vegazana Campus, E-24071 Leon (ES). KUMAR, C., Vasant [IN/US]; North Broad Street, Philadelphia, PA 19140 (US). <b>(74) Common Representative:</b> MERCK & CO., INC.; Patent Dept., 126 East Lincoln Avenue, Rahway, NJ 07065 (US).		<b>(81) Designated States:</b> CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> A METHOD OF TRANSFORMATION OF NOCARDIA  <b>(57) Abstract</b>  A method is disclosed for the high efficiency transformation of species of the genus <i>Nocardia</i> with DNA molecules. DNA vectors for the transformation of genes into <i>Nocardia</i> as well as recombinant <i>Nocardia</i> host cells expressing recombinant genes are disclosed.		

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TITLE OF THE INVENTION

## A METHOD OF TRANSFORMATION OF NOCARDIA

BACKGROUND OF THE INVENTION

5               Several species of Nocardia and the related genus Amycolatopsis are used industrially for the production of antibiotics. Nocardia lactamdurans (Wesseling and Lago, 1981, Developments Ind. Microbiol., 22, pp 641-645) (previously described as Streptomyces  
10 lactamdurans, Stapley et al., 1972 Antimicrob. Agents Chemother., 2, pp 122-131) produces the  $\beta$ -lactam antibiotic cephamycin C (Castro et al., 1985, Appl. Microbiol., Biotechnol., 22, pp 32-40; Cortés et al., 1987, J. Gen. Microbiol., 133, pp 3165-3174) and the polyether efrotomycin. Nocardia mediterranei (recently renamed Amycolatopsis mediterranei)  
15 (Lechevalier et al., 1986, Int., J. Syst., Bacteriol 36, pp 29-37) produces rifamycin and A. orientalis synthesizes vancomycin. The biosynthetic pathway of cephamycin in N. lactamdurans has been extensively studied (Castro et al., 1988, J. Gen., Microbiol., 134, pp 133; Cortés et al., 1987  
20 supra; Láiz et al., 1990, J. Gen., Microbiol, 136, pp 663-671) and the cluster of the cephamycin biosynthetic pathway has been cloned (Coque et al., 1991, mol. Microbiol., 5, pp 1125-1133; Coque et al., 1991, J. Bacteriol., 173, pp 6258-6264; Coque et al., 1993, Mol. Gen. Genet., 236,  
pp 453-458; Coque et al., 1993, EMBO J., 12, pp 631-639). However, the difficulty to transform N. lactamdurans has hampered further studies  
25 on gene disruption and gene amplification.

N. lactamdurans could not be transformed with vectors based on Streptomyces replicons. Since no endogenous circular plasmids occur in N. lactamdurans replicons from different bacteria related to the Nocardia group, e.g. corynebacteria, Rhodococcus fascians,  
30 Amycolatopsis sp and Streptomyces lividans were studied. Polyethylene glycol-assisted transformation of protoplasts (Hopwood et al., 1985 J. Gen. Microbiol., 129, pp 2703-2714) and electroporation have been widely used for transformation of Streptomyces. Another method which uses a combination of polyethylene glycol and alkaline cations to

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transform cells of A. mediterranei was developed by Madon and Hutter (1991) J. Bacteriol., 173, pp 6325-6331.

The current processes for cephamycin production on an industrial scale rely on non-recombinant microbial synthesis systems without the advantages conferred by genetic manipulation of the cephamycin producing organism.

### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 Effect of the age of N. lactamdurans culture in NYG medium on the efficiency of transformation; growth as  $\mu\text{g}$  of cellular DNA per ml (o); number of transformants relative to the best transformation conditions (1) ( $2 \times 10^5$  transformants per  $\mu\text{g}$  of DNA was considered as 100%).

Figure 2 Panels A-D Optimization of different parameters affecting the efficiency of transformation; Panel A, Concentration of polyethylene glycol 1000; Panel B, Concentration of CsCl; Panel C, Concentration of plasmid DNA (pULVK1) per transformation experiment; Panel D, Concentration of competing (calf thymus) DNA.

Figure 3 Formation of the stable plasmid pULVK1 by natural deletion in N. lactamdurans LC411 of plasmid pRL1 is shown; thin line: DNA originating from E. coli plasmids carrying the pBR322 origin of replication (pBR-ori) and the kanamycin resistance gene from Tn5 ( $\text{Km}^R$ ); thick line: DNA from Amycolatopsis sp. containing the pA387 origin of replication (pA-rep); the dashed arc indicates the fragment of DNA which is deleted to form pUVK1.

Figure 4 Improved plasmids for transformation of N. lactamdurans. pULVK2 carries a synthetic polylinker with several unique restriction sites (indicated by asterisks); pULVK3 carries the multiple cloning site from phage M13; pULVK2A contains the apramycin resistance gene ( $\text{Am}^R$ ) in addition to the kanamycin resistance gene ( $\text{Km}^R$ ), and pULAM2 carries the  $\alpha$ -amylase gene (amy) of Streptomyces griseus; all

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these plasmids have the origin of replication of pA387 of Amycolatopsis sp (pA-rep) and the origin of replication of pBR322 (pBR-ori); the S. griseus DNA fragment in pULAM2 containing the amy gene is indicated by double line.

Figure 5 Promoter-probe plasmids pULVK4 and pULVK5 containing the promoterless xylE from Pseudomonas putida in addition to the apramycin (Am<sup>R</sup>) and kanamycin (Km<sup>R</sup>) resistance genes with the origins of replication pA-rep and pBR-ori as shown in Fig. 4.

#### SUMMARY OF THE DISCLOSURE

High transformation efficiency (about 1 to 7x10<sup>5</sup> transformants per µg of DNA) of Nocardia was obtained by direct treatment of mycelium with polyethylene glycol and cesium chloride. A variety of vectors from Streptomyces lividans, Brevibacterium lactofermentum, Rhodococcus fascians and Nocardia (Amycolatopsis) sp. were tested; transformants were obtained with vectors derived from an endogenous plasmid of the strain Amycolatopsis sp DSM 43387. Vectors were constructed carrying the kanamycin resistance gene (kan) as selective marker. The transformation procedure was optimized using one of these vectors (pULVK1) by studying the influence of the age of culture, concentrations of cesium chloride and polyethylene glycol, amount of plasmid DNA, and nutrient supplementations of the growth medium. Versatile bifunctional cloning vectors (pULVK2, pULVK3) were developed by subcloning a multiple cloning site, or a synthetic polylinker containing several unique restriction sites (EcoRV, DraI, BamHI, EcoRI and HindIII). A second marker, the apramycin resistance gene (amr) was added to the vectors (pULVK2A) allowing insertional inactivation of one of the markers while using the second marker for selection. An alternative marker, the amy gene of Streptomyces griseus, was added (PULAM2) which is easily detected by the release of extracellular amylase in transformants carrying this vector. Two promoter probe plasmids pULVK4 and pULVK5 have been constructed, using the promoterless xylE gene as reporter.

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### DETAILED DESCRIPTION OF THE INVENTION

Methods for efficient transformation of S. lividans and several other species of Streptomyces have been developed (Thompson et al., 1982 J. Bacteriol., 151, pp. 668-677; Matsushima and Baltz, 1985 J. Bacteriol., 163, pp. 180-185). However transformation of many species of Streptomyces and other rare actinomycetes of industrial relevance had not been achieved. Efficient plasmid transformation of the  $\beta$ -lactam producer S. clavuligerus has been developed (García-Domínguez et al., 1987 Appl. Environm. Microbiol., 53, pp. 1376-1381), but repeated attempts to transform the cephamycin C producer N. lactamdurans using Streptomyces vectors were unsuccessful since plasmids containing origins of replication from S. lividans, R. fascians or B. lactofermentum do not transform N. lactamdurans. The present invention discloses the successful transformation of N. lactamdurans using vectors derived from the endogenous Amycolatopsis sp DSM43387 plasmid pA387 (plasmid pA387 is described by Lal et al., 1991 Appl. Environm. Microbiol., 57, pp. 665-671). Successful transformation of Nocardia species using the vectors of the present invention is of great interest since the genera Nocardia and Amycolatopsis appear to be closely related (Lechevalier, 1989 In: Bergeys Manual of Systematic Bacteriology, Vol. 4; Williams, Sharpe, and Holt (eds) pp. 2348-2361, Williams and Wilkins, Baltimore, MD).

Polyethylene glycol (PEG)-assisted transformation of protoplasts is well known. The lack of transformation of N. lactamdurans protoplasts even with pULVK1 (described below) appears to be related to the poor DNA uptake of N. lactamdurans protoplasts. Regeneration of the complex cell wall of N. lactamdurans protoplasts might be incomplete, as described also for regeneration of corynebacteria protoplasts apparently due to the need of synthesizing mycolic acids (Martín et al., 1987 Bio/Technology, 5, pp. 137-146).

Electroporation has proved to be useful for transformation of bacteria for which alternative DNA transfer methods did not exist or were inefficient. Electroporation had been applied to transformation of

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corynebacteria (Dunican and Shivnan, 1989 Bio/Technology, 7, pp. 1067-1070) and lactic acid bacteria (Powell et al., 1988 Appl. Environm. Microbiol., 54, pp. 655-660). The inefficient transformation of N. lactamdurans by electroporation prompted us to try direct transformation of entire cells with polyethylene glycol and alkaline cations, a method reported for transformation of Amycolatopsis mediterranei (Madon and Hutter, 1991 J. Bacteriol., 173, pp. 6325-6331). In this method the mycelium does not need any special preparation and the critical steps of protoplast regeneration are avoided.

Several factors were optimized to get high efficiencies of transformation. The age of the culture was critical to get a good transformation efficiency. Efficient transformation was observed during a period of about 20 hours between approximately 20 hours and 40 hours of logarithmic growth. The preferred efficiency was obtained at about 24 hours when the cells are in the logarithmic phase of growth and decreased after about 40 hours probably due to the modification of the cell wall or to the formation of nucleases. In S. clavuligerus a similar effect of the cell growth phase on protoplast transformation was observed (García-Domínguez et al., 1987 *supra*).

The source of plasmid DNA used in transformation was important for successful electroporation of N. lactamdurans. A 40 to 80-fold increase in the efficiency of transformation was obtained when plasmid DNA was isolated from a dam<sup>-</sup>, dcm<sup>-</sup> strain of E. coli instead of E. coli DH5 $\alpha$ , which indicates that the different methylation of the plasmid DNA from E. coli as compared to the normal modification in Nocardia is responsible for the low efficiency of initial transformation. This problem was avoided by the use of an E. coli dam<sup>-</sup> dcm<sup>-</sup> strain or by isolating plasmid DNA directly from previously transformed N. lactamdurans. Strains of E. coli which are dam<sup>-</sup> dcm<sup>-</sup> are commercially available and include but are not limited to JM110. The presence of DNA restriction system in N. lactamdurans may explain the low transformation efficiency obtained by using DNA from heterologous hosts. In fact, a methyl-specific restriction system has been found in the actinomycete Streptomyces avermitilis (MacNeil, 1988 J. Bacteriol., 170,

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pp. 5607-5612) and similar restriction systems are likely to occur in many other actinomycetes.

One important finding was the observation of the deletion of a constant 4.0 kb fragment from plasmid pRL1 which gave rise to the stable plasmid pULVK1. A similar deletion of unstable corynebacterial plasmid pULRS61 which originated the stable plasmid pUL330 and 340 was found in B. lactofermentum (Santamaría et al., 1984 J. Gen. Microbiol., 130, pp. 2237-2246; Santamaría et al., 1985 J. Bacteriol., 162, pp. 463-467). Such deletions occur frequently with plasmids that contain short repeats of a certain sequence (Albertini et al., 1982 Cell, 29, pp. 319-328; Murray et al., 1989 Gene, 85, pp. 283-291; Criado et al., 1993 Gene, 126, pp. 135-139) or that replicate via the rolling circle mechanism (Fernández et al., 1994). The deleted plasmid pULK1 gave very high efficiencies of transformation (up to  $2 \times 10^5$  transformants per  $\mu\text{g}$  of DNA) as compared to the undeleted pRL1 form which indicates that many cells transformed with the undeleted form were probably unable to replicate the plasmid in an stable form and it was lost from the cells.

The copy number of pULVK1 that was observed (20-30 copies/cell) is lower than the copy number reported for pRL1 (about 90 copies/cell) (Lal et al., 1991 *supra*) possibly due to less recognition of the origin or replication of the Amycolatopsis sp. plasmid by the N. lactamdurans plasmid replication machinery. The stable plasmid pULVK1 has served as the starting point to construct a variety of plasmids with two selective markers (Figs. 3 and 4) and promoter-probe vectors (Fig. 5) for use in N. lactamdurans. These vectors are very useful instruments for advanced molecular genetics applications of N. lactamdurans. A variety of selective markers are suitable for use in the present invention. Suitable selectable markers are known in the art and include but are not limited to kanamycin resistance gene, thiostrepton resistance gene, erythromycin-resistance gene, apramycin resistance gene and phleomycin-resistance gene. In addition, a variety of promoters are suitable for use in the present invention for expression of recombinant genes. Suitable promoters include but are not limited to the tip



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(thiostrepton induced protein) promoter, the kan (kanamycin-resistance gene) promoter, the cefD (isopenicillin N epimerase) promoter, and the mel (melanine biosynthesis) promoter. While the microorganism N. lactamdurans is used to demonstrate the present invention, it is readily  
5 apparent to those of ordinary skill in the art that other species of *Nocardia* are suitable for use. Other species of *Nocardia* include but are not limited to *Nocardia amarae*, *Nocardia brevicatena*, *Nocardia carneae*, *Nocardia cellulans*, *Nocardia (Amycolatopsis) mediterranei*, and *Nocardia (Amycolatopsis) orientalis*.

10 The following examples are provided as illustrative of the present invention without however, limiting the same thereto.

### EXAMPLE 1

#### Selection of antibiotic resistance markers

The sensitivity of N. lactamdurans to different antibiotics was tested in S27M (Madon and Hutter, 1991 supra) or modified ELR medium (Wesseling and Lago, 1981 supra). N. lactamdurans was  
15 resistant to hygromycin and viomycin (MIC higher than 100 µg/ml) or chloram-phenicol (MIC higher than 75 µg/ml) but it was sensitive to  
20 thiostrepton (5 µg/ml) phleomycin (15 µg/ml), kanamycin (30 µg/ml) or apramycin (25 µg/ml). Thiostrepton-resistant mutants appeared spontaneously with high frequency. Therefore, the thiostrepton resistance (tsr) marker was not used. In addition, thiostrepton is known  
25 to have multiple regulatory effects on N. lactamdurans and other actinomycetes (Kumar et al., 1993; García-Domínguez et al., 1991 Antimicrob Agents Chemother., 35, pp. 44-52). Although *Nocardia* is sensitive to phleomycin, the antibiotic cannot be used for primary  
30 selection since it appears that components of S27M medium affects the antibiotic activity. Kanamycin was used as the first selective marker because of the high level of resistance that was conferred by the kan gene of transposon Tn5 and the availability of the antibiotic. Apramycin or phleomycin resistance genes were used as secondary markers. The unique XhoI site in the amr gene can be used for cloning XhoI

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compatible fragments what results in the insertional inactivation of this selective marker.

## EXAMPLE 2

5

### Transformation procedure

A seed culture of N. lactamdurans LC 411, a stable variant isolated from the wild type NRRL 3802 was grown by inoculating 1.5 ml of stock cell suspension (in 20% glycerol) into NYG medium [Ginther, C.L., 1979, Antimicrob. Agents Chemother., 15, pp. 522-526] supplemented with 0.85% MgCl<sub>2</sub>, and incubated for 36 hours at 30°C and 250 rpm in an orbital shaker. After growth, 5 ml of this culture were used to inoculate 100 ml of the same medium containing 0.1% Tween 80 in 500 ml baffled Erlenmeyer flasks, and the incubation was carried out in the same conditions. Cells (10 ml aliquots) were collected at different times by centrifugation, resuspended in 5 ml of 20% glycerol and preserved at -20°C until use.

For transformation, cells were pelleted by centrifugation, washed twice with the same volume of sterile TE buffer [20 mM TRIS, 1mM EDTA, pH8.0], and finally with a 25 mM TRIS-HCl (pH 8.0) solution, and then resuspended in one tenth of the original volume in the same buffer. The transformation mixture consisted of 50 µl of 4M CsCl; 1 µl of 1M MgCl<sub>2</sub>; 5.0 µl of sonicated calf thymus DNA (5 µg/µl) and plasmid DNA in 2.5 µl volume. The total volume was made up to 100 µl by adding the N. lactamdurans cell suspension. After that, 100 µl of 80% PEG 1000 (Khock Light, UK) were added to a final concentration of 40%. The components of the transformation mixture were mixed carefully by pipetting up and down, incubated at 30°C for 1 hour, and then at 42°C for 10 minutes.

After the incubation, aliquots of the transformation mixture were plated onto S27M media plates (Madon and Hutter, 1991), previously dried in a hood for about 2 to 3 hours, by mixing the transformed cells with 2-3 ml of an overlay of melted R2L agarose (0.7%). PEG elimination from the transformation mixture is not

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required, since it is not toxic for the microorganisms and does not have any influence on the final result of the transformation. In some experiments the transformation mixture was diluted in R2L liquid medium [or in P buffer (Hopwood et al., 1985)] and then plated in the same way.

Plates were briefly dried before they were incubated at 30°C. Kanamycin (75 µg/ml in water solution) or apramycin (50 µg/ml) was added after 20 hours of incubation. Transformants began to appear about 72 to 96 hours after adding the antibiotic. Single colonies were picked up for further analysis.

#### Electroporation

Cells grown in NYG medium as indicated above were harvested at different times and then washed 5 times with milli-Q sterile water and finally resuspended in one fiftieth of the original volume and preserved in aliquots until use. Cell suspensions (75 µl aliquots) were mixed with 1-2 µl of plasmid DNA and then transferred into a chilled electroporation cuvette, and exposed to a single pulse of varying field strengths (5 to 12.5 kv/cm) and pulse duration from 2.3 to 6.6 milliseconds in a BioRad apparatus. After electroporation, cells were added to 0.5 ml of NYG medium and incubated with agitation at 30°C for about 3-6 hours and then plated onto plates containing the antibiotic (kanamycin, 75 µg/ml or apramycin, 50 µg/ml).

### EXAMPLE 3

#### Protoplast transformation and electroporation

Initially, transformation of N. lactamdurans protoplasts was tried with vectors pIJ702 or pIJ699 from S. lividans (Hopwood et al., 1985 supra; Matsushima et al., 1987 supra), pUL340, pULRS8 and pULMJ600 of B. lactofermentum (Santamaría et al., 1984 supra; 1985 supra; Martín et al., 1990 pp. 283-292, In: Proceeding Of The 6th International Symposium on Genetics of Industrial Microorganisms, Heslot, Davies, Florent, Robichon, Durand and Penasse (eds), Societé Francaise de Microbiologie, Paris), pRL1 of Amycolatopsis sp. and

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pULRE1, pULRE2 and pULRE3 of Rhodococcus fascians. No transformants were obtained with any of the plasmids probably due to the inability of these plasmids to replicate in N. lactamdurans or to the low efficiency of protoplast DNA uptake, although N. lactamdurans can be easily protoplasted and regenerated in the modified ELR or S27M media described for this purpose.

A variety of electroporation conditions (with the same plasmids) were tested by varying the field strength from about 5 to 12.5 kv/cm and pulse duration from about 2.3 to 6.6 milliseconds. The results of the electroporation were very poor (0.1-1 transformants per  $\mu\text{g}$  of DNA) with plasmid pRL1 and did not improve by growing the cells in presence of glycine (0.5-1%) and/or Tween-80, or by pretreatment of the mycelium with lysozyme (100  $\mu\text{g}/\text{ml}$ ) for 5 min. No transformants were obtained after electroporation with plasmids from corynebacteria, R. fascians or S. lividans.

#### EXAMPLE 4

##### Initial polyethylene glycol and cesium chloride assisted transformation of intact cells

A successful transformation of N. lactamdurans with plasmid pRL1 obtained from E. coli DH5 $\alpha$  was obtained when intact cells were incubated with plasmid DNA in presence of polyethylene glycol and CsCl. The low initial efficiency (1 to 10 transformants per  $\mu\text{g}$  of DNA) was significantly increased (40-80 transformants per  $\mu\text{g}$  of DNA) when pRL1 was obtained from E. coli JM110 (a dam<sup>-</sup>, dcm<sup>-</sup> strain) instead of E. coli DH5 $\alpha$ .

The age of the N. lactamdurans culture was very important to get the highest transformation efficiencies. Two to five-fold higher efficiencies (up to about 400 transformants per  $\mu\text{g}$  of DNA) were obtained by using 24 hour mycelium than when 36 hour old mycelium was used. Cells from cultures of about 48-72 hours were more difficult to transform (Fig. 1) but transformants were obtained nonetheless. The best efficiency of transformation was obtained when the culture was in

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the exponential phase of growth, about 8 to 10 hours before the organism reaches the stationary phase.

### EXAMPLE 5

#### Optimization of polyethylene glycol, cesium chloride, Tween 80 and DNA concentrations

Initial studies indicated that growth of *N. lactamdurans* in NYG medium supplemented with  $\text{MgCl}_2$  (8.5 g/l) and Tween -80 (0.1%) supported a higher transformation efficiency than TSB with the same added components. Other compounds such as glycine (0.5-1%) (a known inhibitor of cell wall biosynthesis), alone or in combination with Tween-80, and/or  $\text{MgCl}_2$  always yielded a lower number of transformants.

The addition of PEG to the transformation mixture was strictly required. No transformants were obtained in the absence of PEG. A good transformation efficiency was obtained in a narrow range of PEG 1000 concentration (from about 30- 48%); the optimal efficiency was consistently observed at about 40% PEG (Fig. 2A).

Another important factor to obtain efficient transformation was the  $\text{CsCl}$  concentration. Relatively high concentrations of  $\text{CsCl}$  (above about 0.4 M) were required. The optimal efficiency of transformation was observed at about 1 M concentrations of this salt, although a high number of transformants was also obtained at lower or higher  $\text{CsCl}$  concentrations (Fig. 2B). Substitution of  $\text{CsCl}$  either by  $\text{RbCl}$ ,  $\text{LiCl}$  or  $\text{KCl}$  at 1 M concentration, yielded only 36% (for  $\text{RbCl}$ ) or 0% (for  $\text{KCl}$  or  $\text{LiCl}$ ) of the transformants obtained with  $\text{CsCl}$  (Table 1). Likewise substitution of  $\text{Mg}^{2+}$  by  $\text{Ca}^{2+}$  at the same concentration (about 1 M) yielded about 30-40% transformants as compared to control transformations with  $\text{Mg}^{2+}$ .

### TABLE 1

Effect of different alkaline salts on the transformation of cells of *N. lactamdurans*

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	Alkaline salts	Efficiency of transformation	
		Number of transformants/ $\mu$ gDNA	%
5	CsCl 1M	$6.7 \times 10^5$	100
	RbCl 1M	0	0
	LiCl 1M	$2.4 \times 10^5$	36
	KCl 1M	0	0
10	CsCl 1M, CaCl <sub>2</sub> 0.1M (substituting for MgCl <sub>2</sub> 0.1 M)	$2.6 \times 10^5$	38

0.1 M MgCl<sub>2</sub> was used in all experiments except in the last transformation in which it was replaced by 0.1 M CaCl<sub>2</sub>.

15 Concentrations of plasmid DNA higher than about 1  $\mu$ g per transformation reaction were required to obtain good transformation efficiencies and saturation of the transformation reaction was obtained by using 2  $\mu$ g of plasmid DNA (Fig. 2C). The concentration of calf thymus DNA used as a carrier was not very important although the  
20 transformation efficiency decreased when the amount used was higher than 25  $\mu$ g per reaction (Fig. 2D). Using the optimized transformation conditions, efficiencies of approximately 1 to  $7 \times 10^5$  transformants/ $\mu$ g of DNA were routinely obtained.

25

### EXAMPLE 6

#### Isolation of a stable deleted plasmid from *N. lactamdurans*

pRL1 was relatively unstable in *N. lactamdurans*. About 70-75% of the transformants obtained with pRL1 isolated from *E. coli*  
30 JM110 showed a deleted plasmid of about 6.0 kb. A similar proportion (about 80%) of the transformants obtained with plasmid pRL1 isolated for *E. coli* DH5 $\alpha$  also yielded the same deleted plasmid.

One of the deleted plasmids, named pULVK1, was mapped in detail by restriction analysis (Fig. 3). A region of 4.0 kb of pRL1 has been deleted (Fig. 3). The deleted plasmid pULVK1 retained the origins

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of replication of E. coli, and Amycolatopsis sp, and the kanamycin resistance marker. Plasmid pULVK1 was stable and could be propagated in either E. coli or Nocardia lactamdurans and has been routinely used in cloning experiments. It has an approximate copy number of 20-30 copies/cell.

DNA fragments ranging from about 2-10 kb were successfully cloned in the polylinker sites without affecting the stability of the plasmid.

### EXAMPLE 7

#### Development of improved cloning vectors

New improved cloning vectors were developed by replacing the EcoRV-HindIII fragment of pULVK1 with a synthetic linker with several cloning sites (EcoRV, DraI, BamHI, SacI, EcoRI, XbaI and SstI) resulting in plasmid pUVK2 (5.5 kb) (Fig. 4). In a different strategy the multiple cloning site of pBluescript KS(+) was subcloned as a 0.45 kb PvuII fragment. The resulting plasmid, pULVK3, has a size of 6.4 kb (Fig. 4). These plasmids can be easily isolated from N. lactamdurans using the alkaline SDS-lysis method of plasmid isolation from Streptomyces (Kieser, 1984 Plasmid, 12, pp. 19-36).

#### Construction of vectors with two markers

The apramycin resistance gene was isolated as a 1.2 kb EcoRI-PstI fragment from cosmid pKC505, cloned in the EcoRI-PstI sites of pIJ2921 [Janssen and Bibb, 1993, Gene, 124, pp. 133-134] and subcloned as a EcoRI-BamHI fragment into pULVK2 yielding plasmid pULVK2A (Fig. 4).

A different marker was introduced at the BamHI site of pULVK2 by inserting the amy gene of Streptomyces griseus from pULVK2 which is efficiently expressed in N. lactamdurans. The new vector with the kan and amy markers was named pULAM2 (Fig. 4). Clones of N. lactamdurans transformed with pULAM2 were easily

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detected in starch based minimal medium due to the surrounding halo of degradation of starch when exposed to iodine vapors.

By replacing the HindII-EcoRV fragment of pULVK1 by the 4.4 kb HindIII-EcoRV region of pIJ699 [Kieser and Melton, 1988, Gene, 65, pp. 83-91] containing the origin of replication of S. lividans pIJ101 [Kieser et al., 1982, Mol. Gen. Genet., 185, pp. 223-228], a new multifunctional vector pULVKT3 able to replicate in E. coli, N. lactamdurans and several Streptomyces species was obtained, using the kanamycin resistance gene as selective marker in the three microorganisms.

pULVKT3 was used successfully to transform S. clavuligerus and still retains the E. coli DNA fragment unlike other Streptomyces plasmids such as pIJ699 in which the E. coli part is deleted when introduced in S. clavuligerus. The multifunctional vector pULVKT3 is useful to transfer directly cephamycin biosynthetic genes from N. lactamdurans to S. clavuligerus and vice versa.

#### Promoter probe vectors

The promoter-less xylE of Pseudomonas putida was subcloned as a 1.5 kb BglII fragment from the Streptomyces promoter-probe vector pIJ4083 [Clayton and Bibb, 1990, Nucl. Acids Res., 18, pp. 1077] in both orientations in the BamHI site of pULVK2A yielding the 8.4 kb promoter probe vectors pULVK4 and pULVK5 (Fig. 5).

The usefulness of these vectors was confirmed by subcloning the lat gene promoter of the cephamycin cluster (Coque et al., 1991 J. Bacteriol., 173, pp. 6258-6264) as a HindIII-BamHI fragment in pULVK5 and observing the formation of yellow color in presence of catechol (0.5%). In random cloning of fragments containing promoters it is advisable to replicate the transformants into either MEY or a minimal medium before testing for the yellow color, to avoid the interference of the pink pigment produced by N. lactamdurans in complex media. A minimal medium containing isoleucine as the sole carbon source was found to be the best since in this medium N. lactamdurans grows as white



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colonies and the yellow color formation after spraying with catechol was easily observed.

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WHAT IS CLAIMED IS:

1. A method for transferring DNA into a species of Nocardia, comprising the steps:

- 5 (a) culturing a species of Nocardia in a suitable medium to logarithmic growth phase;  
(b) harvesting the cells;  
(c) incubating the cells in a suitable DNA-transformation mixture;  
and  
10 (d) culturing the cells from step (c) in a suitable medium.

2. The method of claim 1 wherein the DNA-transformation mixture of step (c) comprises at suitable concentration CsCl; MgCl<sub>2</sub>; PEG; and DNA to be transferred into the cells.

3. The method of claim 2 wherein the concentration of CsCl is approximately 0.5M to approximately 1.2M.

4. The method of claim 2 wherein the concentration of MgCl<sub>2</sub> is approximately 0.075M to approximately 0.125M.

5. The method of claim 2 wherein the PEG has a molecular weight of about 1000 and the PEG concentration is approximately 30% to approximately 50%.

6. The method of claim 1 wherein the suitable medium of step (d) is favorable for growth of cells which contain the transferred DNA.

7. The method of claim 6 wherein the medium contains an agent for the selection of cells which contain the transferred DNA.

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8. The method of claim 7 wherein the medium contains an antibiotic selected from the group consisting of thiostrepton, kanamycin, apramycin, phleomycin and erythromycin.

5 9. A plasmid for transformation of a species of *Nocardia* comprising:

- (a) one or more origin of replication;
- (b) at least one site for the insertion of a recombinant transcription unit; and
- 10 (c) optionally containing a selectable marker gene.

10 10. The plasmid of claim 9 wherein at least one origin of replication is from a genus of a microorganism selected from the group consisting of *Ammycolatopsis*, *Streptomyces*, *Brevibacterium*  
15 *Rhodococcus*, *Nocardia*, and *Bacillus*.

11. The plasmid of claim 9 wherein the selectable marker gene confers antibiotic resistance.

20 12. The plasmid of claim 11 wherein the selectable marker gene confers resistance to antibiotics selected from the group consisting of kanamycin resistance gene, thiostrepton resistance gene, erythromycin-resistance gene, apramycin resistance gene and  
25 phleomycin-resistance gene.

13. A plasmid for transformation of a species of *Nocardia* wherein said plasmid is selected from the group consisting of pULVK1, pULVK2, pULVK2A, pULVK3, pULAM2, pULVK4 and pULVK5.

30 14. A recombinant microorganism containing the plasmid of Claim 9 wherein said microorganism is a species of *Nocardia* selected from the group consisting of *N. lactamdurans*, *Nocardia amarae*, *Nocardia asteroides*, *Nocardia autrophica*, *Nocardia brasiliensis*,

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*Nocardia brevicatena*, *Nocardia carnea*, *Nocardia cellulans*, *Nocardia*  
(*Amycolatopsis*) *mediterranei*, and *Nocardia* (*Amycolatopsis*) *orientalis*.

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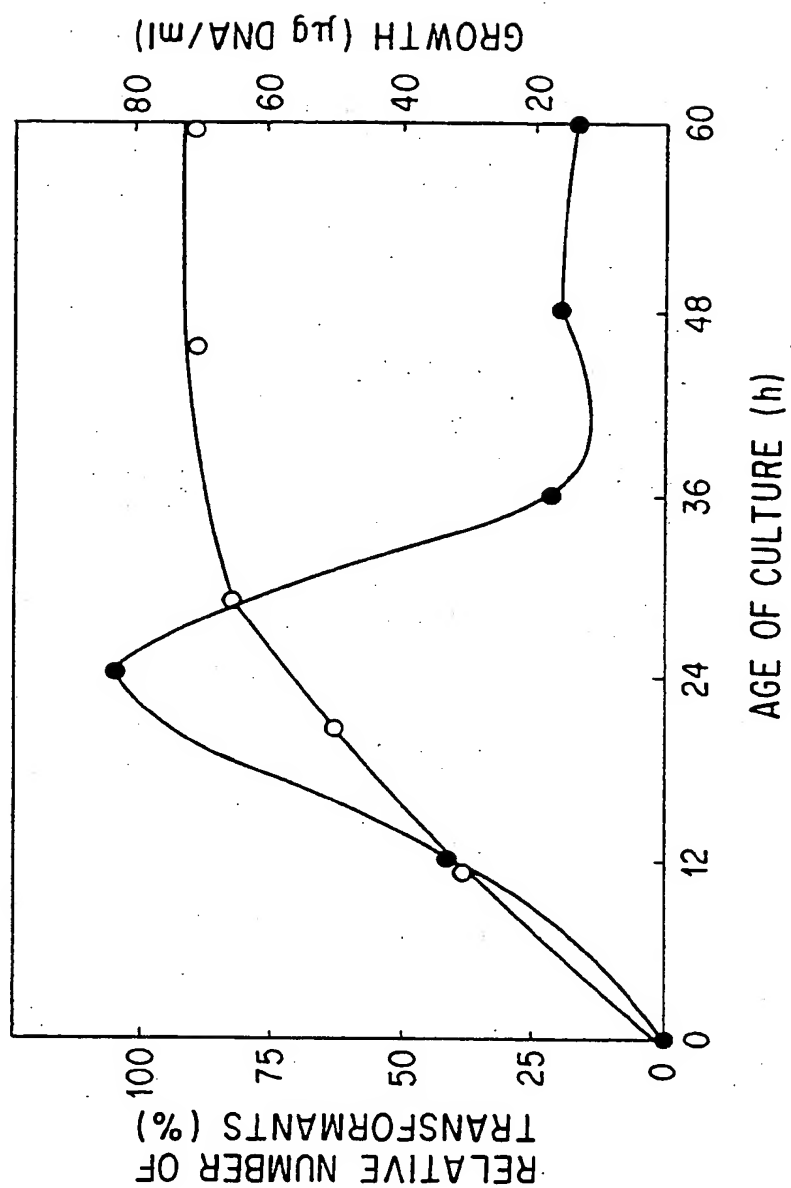


FIG. 1

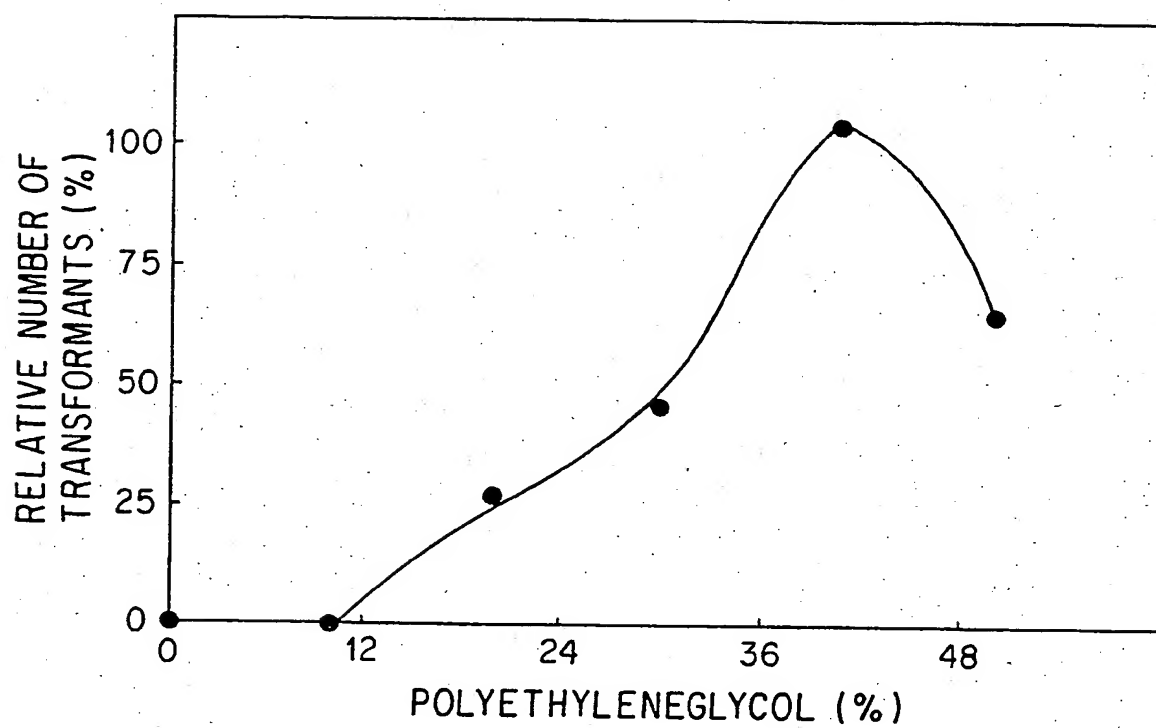


FIG. 2A

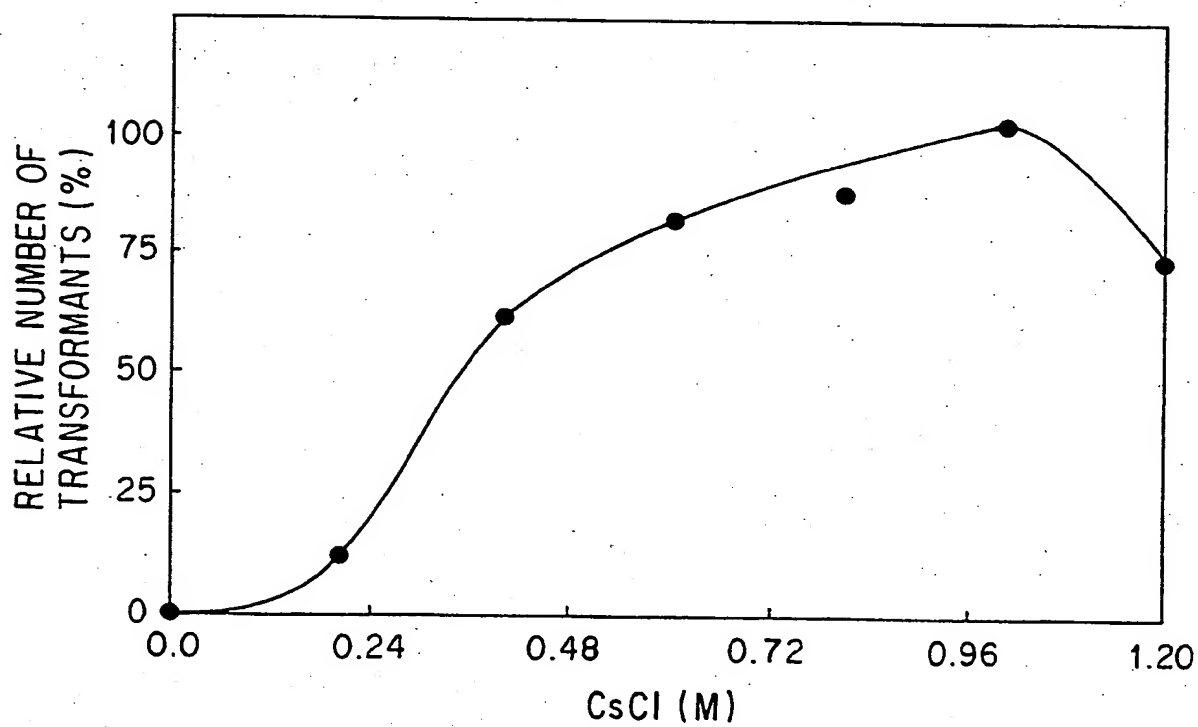


FIG. 2B

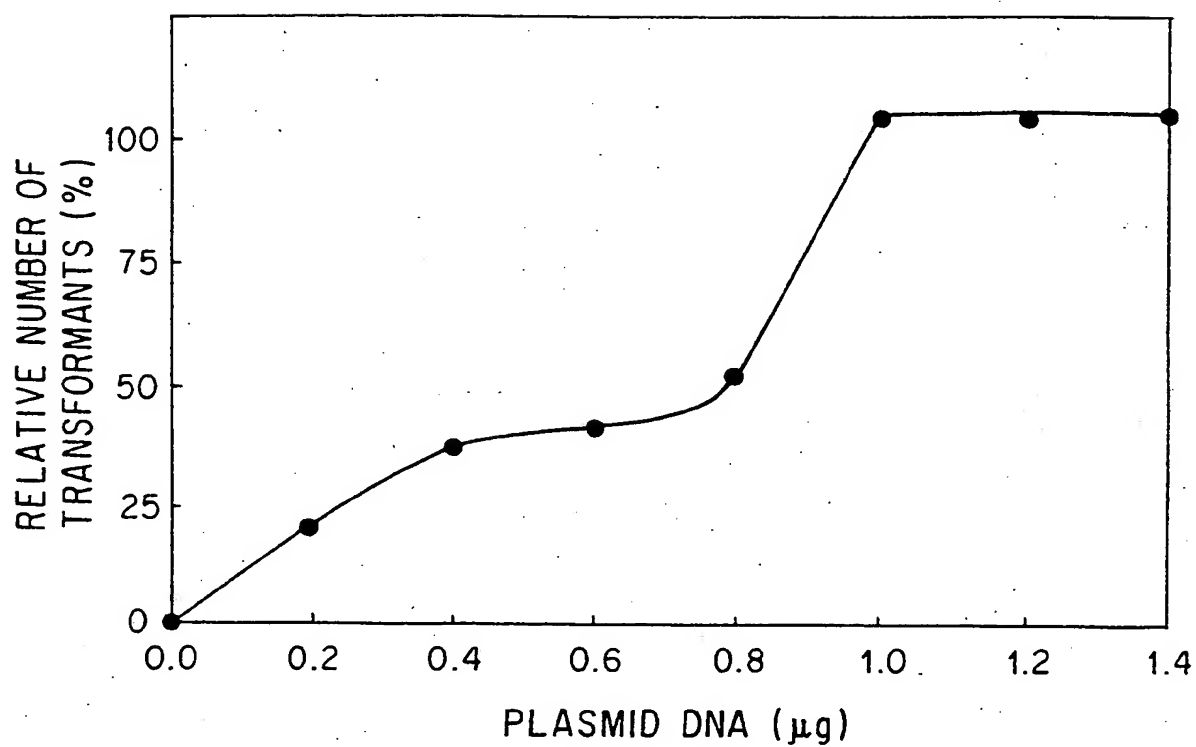


FIG. 2C

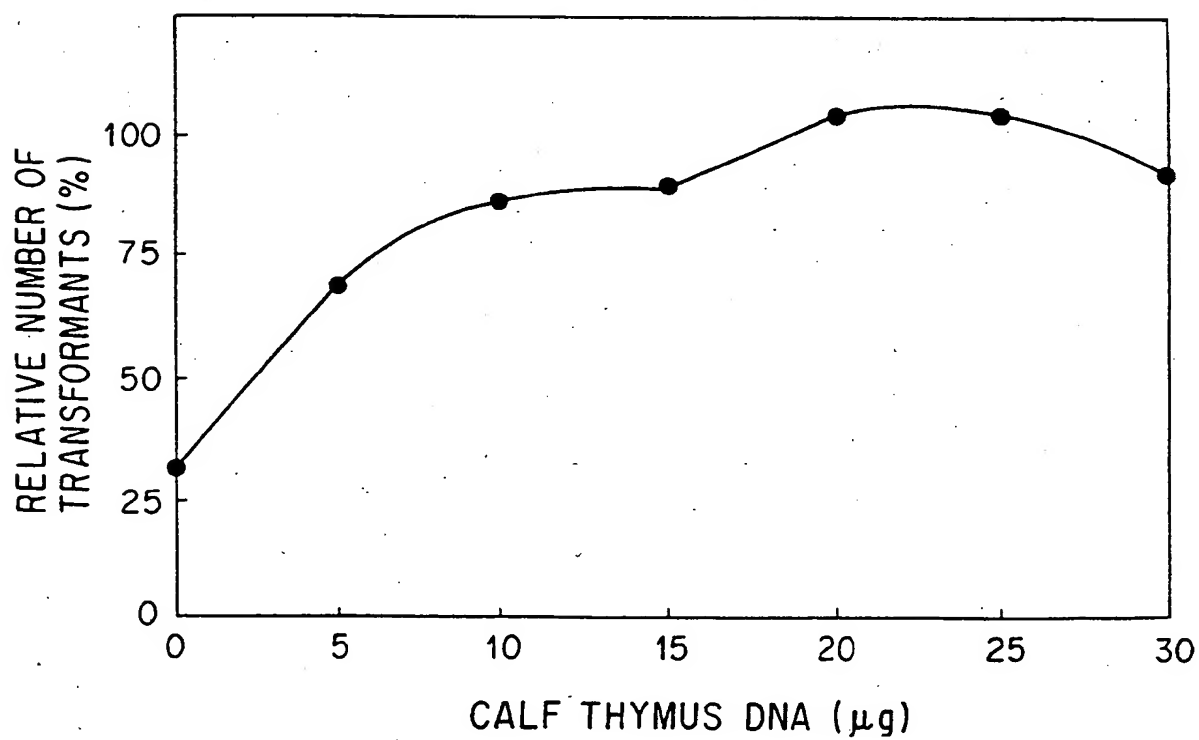


FIG. 2D

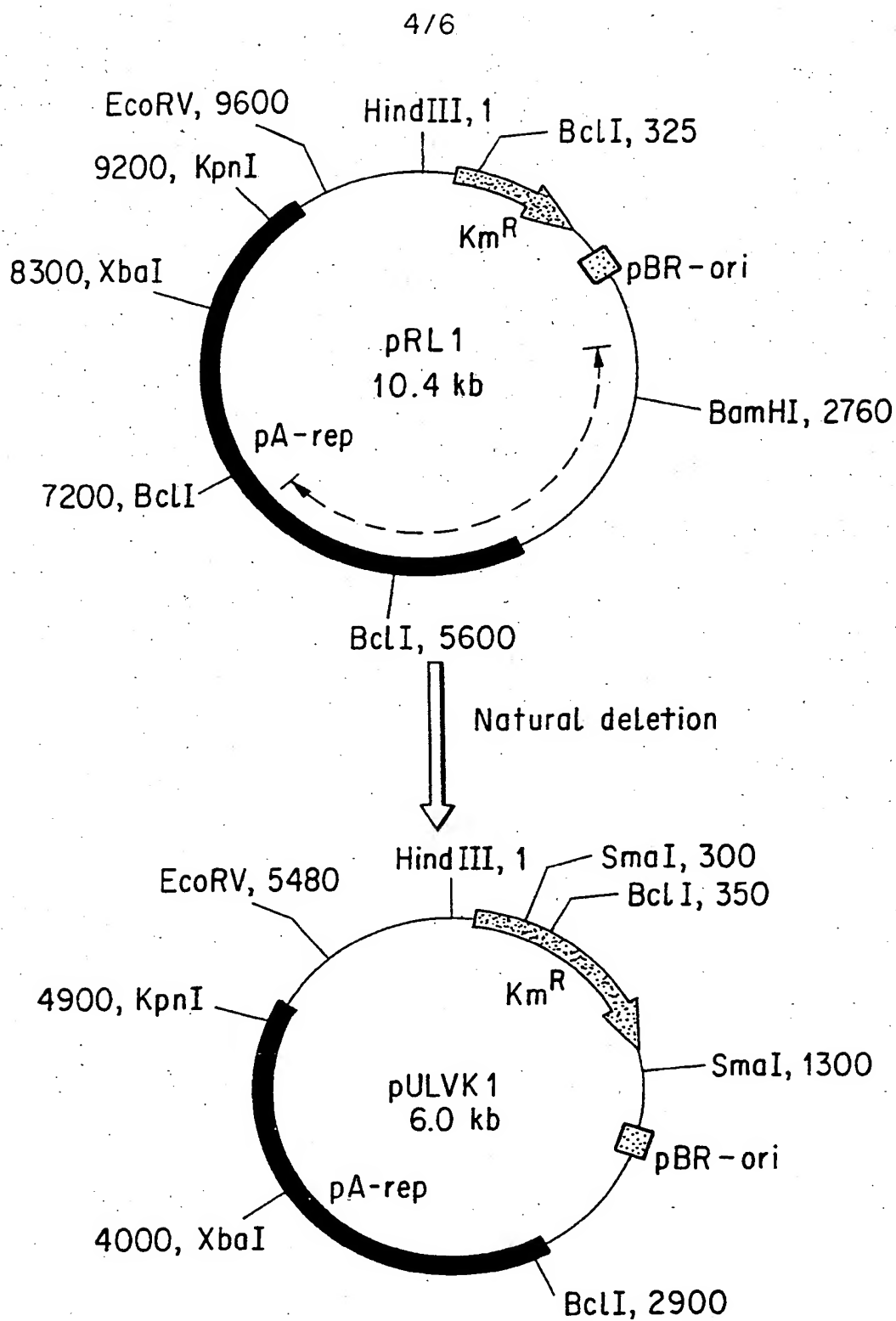


FIG. 3



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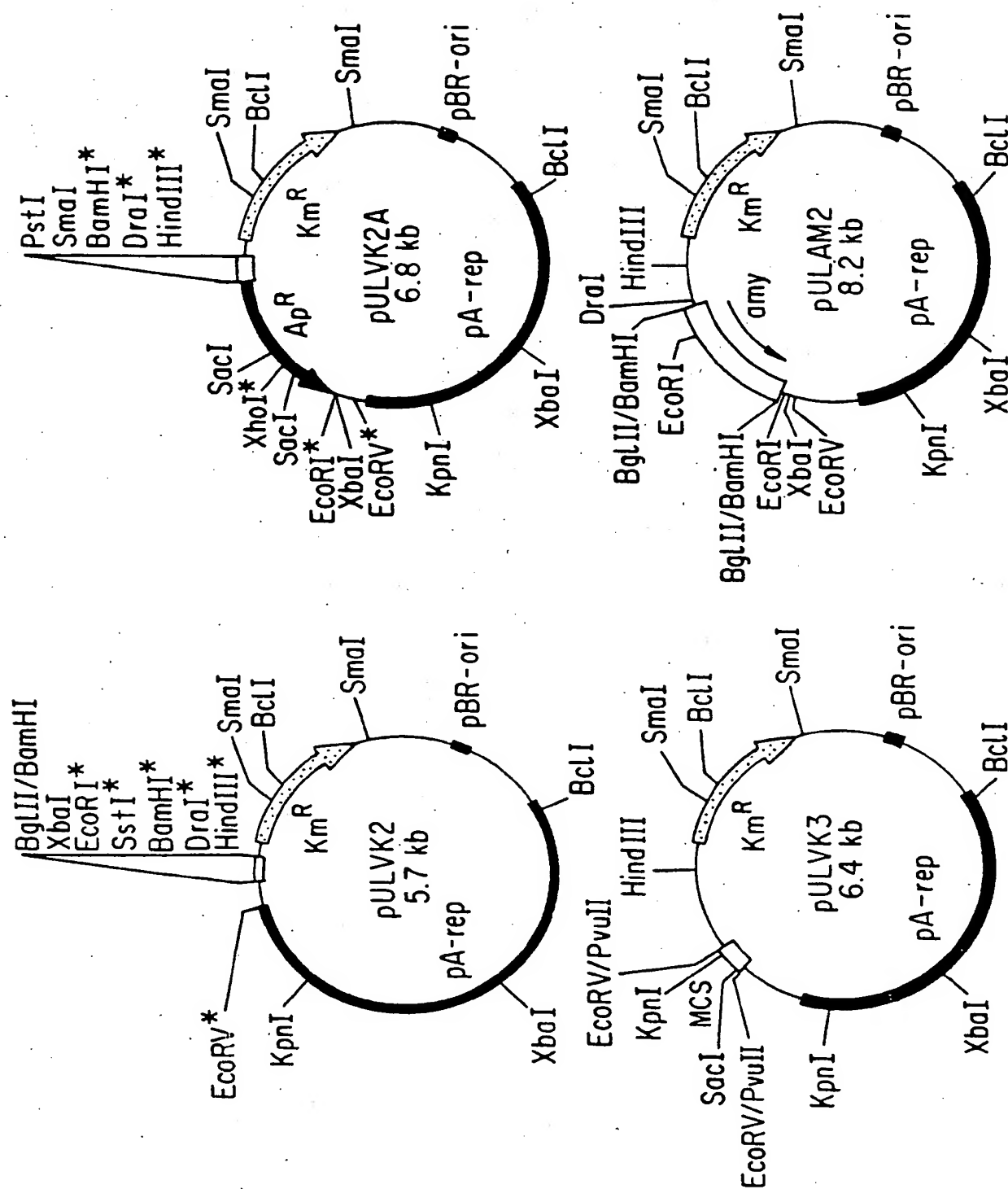


FIG. 4

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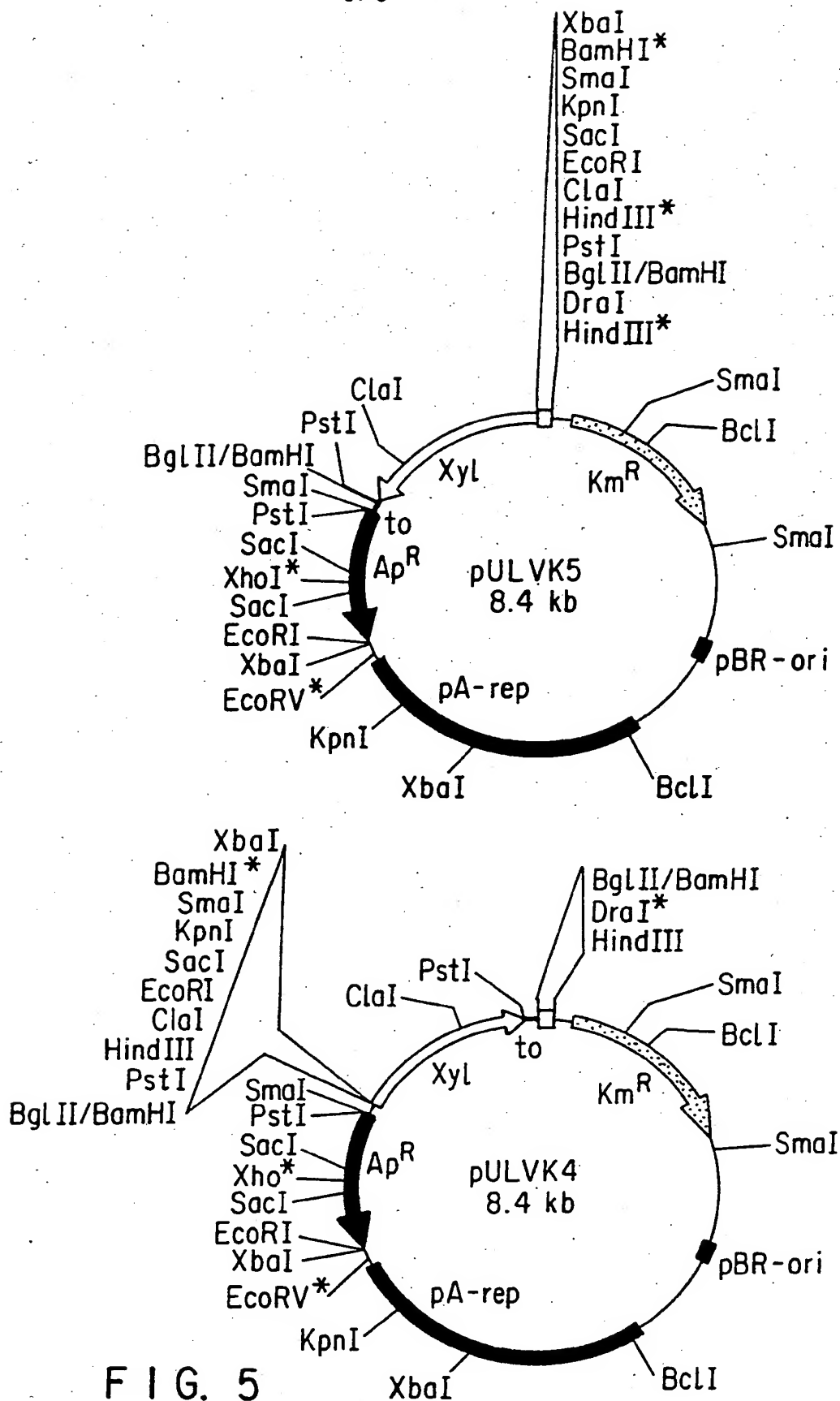


FIG. 5

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/02491

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12N 1/21, I5/64, 15/74

US CL : 435/69.1, 172.3, 172.1, 252.3, 252.32, 320.1

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 172.3, 172.1, 252.3, 252.32, 320.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Biosis, World Patents Index

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,791,064 (FARE ET AL.) 13 DECEMBER 1988, col. 11 line 25 - col. 18 line 67.	1-12, 14
Y	Journal of Bacteriology, Vol. 173, No. 20, issued October 1991, Madon et al., "Transformation system for <i>Amycolatopsis (Nocardia) mediterranei</i> : Direct transformation of mycelium with plasmid DNA", pages 6325-6331, see entire document.	1-14
Y	Journal of Bacteriology, Vol. 169, No. 5, issued May 1987, Matsushima et al., "Efficient transformation of <i>Amycolatopsis orientalis (Nocardia orientalis)</i> protoplasts by <i>Streptomyces</i> plasmids", pages 2298-2300, see entire document.	9-12, 14

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 25 MAY 1995	Date of mailing of the international search report 02 JUN 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer NANCY T. VOGEL Telephone No. (703) 308-0196

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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/02491

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Applied and Environmental Microbiology, Vol. 57, No. 3, issued March 1991; Lal et al., "Construction of a hybrid plasmid capable of replication in <i>Amycolatopsis mediterranei</i> ", pages 665-671, see entire document.	13
Y	Maniatis et al., "Molecular Cloning, A Laboratory Manual", published 1982 by Cold Spring Harbor Laboratory, page 4, see entire page.	13

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